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Vaccination with circulating exosomes in autoimmune uveitis prevents recurrent intraocular inflammation

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ABSTRACT

Background: Exosomes participate in intercellular communication and act as important molecular vehicles in the regulation of numerous physiological and pathological processes, including autoimmune development. The role of circulating exosomes in the development of autoimmune uveitis is unknown. In this study, using the rat model of experimental autoimmune uveitis which has clinical and histologic features of pan uveitis in man, we evaluated the immunoregulatory function of circulating exosomes.

Methods: Experimental autoimmune uveitis was induced in Lewis rats either immunized with interphotoreceptor retinoid-binding protein R16 peptides or injected with activated R16-specific T cells. The disease incidence and severity were examined by indirect fundoscopy and flow cytometry. Circulating exosomes were isolated from peripheral blood of naïve and day 14 R16 immunized Lewis rats. The effect of exosomes on specific T cells were evaluated by R16-specific T cell proliferation, cytokine production and recurrent uveitis induction.

Results: Circulating exosomes derived from active immunized uveitis rats selectively inhibited immune responses of R16-specific T cells *in vitro*. Vaccination of naïve rats with these exosomes reduced the incidence of recurrent uveitis in an antigen specific manner. Antigen specific uveitogenic T cells reduced IFN-□production and increased IL-10 after vaccination.

Conclusion: Circulating exosomes in autoimmune uveitis have the potential to be a novel treatment for recurrent autoimmune uveitis.

Key Words: Immunoregulation, Inflammation, Uveitis, exosomes, autoimmune disease,

1. INTRODUCTION

Exosomes are nano-sized vesicles about 30–100 nm in diameter and are released by various cell types upon fusion of multivesicular bodies (MVB) with the plasma membrane. Consequently, exosomes contain the proteins, lipids and RNA, including mRNA and miRNA, of their parent cells (1). The effect of exosomes from different cell types varies since the composition of their functional molecules changes depending on their cell of origin. Exosomes are released into every circulating body fluid, including blood (plasma, serum), saliva, milk, and urine. Their biological information can be delivered to target cells throughout the body, and act as regulatory elements for numerous physiological and pathological processes, including autoimmune disease (2). For example, circulating exosomes in the blood from multiple sclerosis (MS) patients, a T cell-mediated autoimmune disease of the central nervous system (CNS), inhibited induction of human $Foxp3^+$ regulatory T (Treg) cells. This was accomplished via exosomal let-7i miRNAs, which target insulin like growth factor 1 receptor (IGF1R) and transforming growth factor beta receptor 1 (TGFBR1) (3). Human lung transplant recipients undergoing rejection had circulating exosomes with lung self-antigens, K-alpha 1 Tubulin and Collagen V. Immunization of C57BL/6 mice with these exosomes induced obliterative airway disease (HEI-OAD) (4). Circulating exosomes isolated from murine experimental autoimmune myocarditis (EAM) initiated T cell pathologic immune responses and enhanced EAM via cargo miR-142 (5).

Autoimmune uveitis is a heterogeneous group of diseases characterized by non-infectious inflammation of the uveal tract (i.e., iris, ciliary body, choroid) or adjacent ocular structures (e.g., vitreous, retina, optic nerve) (6). The disease course can be acute, chronic or recurrent (7). Acute uveitis can spontaneously resolve, whereas chronic and recurrent uveitis often result in significant damage to the eye, such as cataract and cystoid macular edema of the retina. As a result, uveitis is one of the major causes of visual disability and accounts for up to 15% of visual disability in the Western world (8). Studies from uveitis patients and animal models of autoimmune

uveitis (EAU) suggest that the disease is a Th1 and/or Th17 cell-mediated immune response to ocular autoantigens, such as interphotoreceptor retinoid-binding protein (IRBP) and S-antigen (S-Ag). These antigens are immunogenic and can result in antigen specific T-cell mediated uveitis in rodents and other species (9-14). It has been recently reported that human plasma exosomes in Vogt-Koyanagi-Harada (VKH) disease, a non-infectious inflammatory uveitis, contain inflammation associated proteins that are upregulated during the active stage of the disease (15). However, the role of circulating exosomes in the development of autoimmune uveitis still needs to be clarified. Importantly, non-circulating exosomes such as those derived from human mesenchymal stem cells (16, 17) and from *ex-vivo*-generated IL-35-producing regulatory B-cells (18) have been demonstrated to ameliorate EAU in both rats and mice.

In our study, we isolated circulating exosomes from the blood of EAU rats induced by immunization with IRBP R16 peptides and evaluated their ability to regulate R16-specific T cell responses. Surprisingly, we found that exosomes derived from EAU (EAU-Exo) rats selectively inhibited immune responses of R16-specific T cells *in vitro*. We then pre-vaccinated naïve Lewis rats with EAU-Exo and induced recurrent EAU in these recipients. Vaccination with EAU-Exo reduced the frequency and severity of recurrent EAU. Antigen specific uveitogenic T cells reduced IFN-□production and increased IL-10 after vaccination. Our results suggest the possibility of utilizing autologous circulating exosomes as a vaccine for the suppression of antigen-specific inflammation in patients with recurrent autoimmune uveitis.

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2. METHODS

2.1 Animals and reagents

Pathogen-free female Lewis rats (5-6 weeks old) purchased from Harlan Sprague-Dawley (Indianapolis, IN) were housed and maintained in the animal facilities of the University of Louisville. All animal studies conformed to the Association for

Research in Vision and Ophthalmology statement on the use of animals in ophthalmic and vision research. The protocol (#17095) was approved by the Institutional Animal Care and Use Committee of the University of Louisville and institutional guidelines regarding animal experimentation were followed.

All T cells were cultured in complete RPMI 1640 medium (CM) which was comprised of RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Gibco), 5 x 10^{-5} M 2-mercaptoethanol, and penicillin/streptomycin (100 μg/ml). Incomplete Freund's adjuvant (IFA) was obtained from Sigma. Mycobacterium H37Ra was obtained from Difco (Detroit, MI). Bovine IRBP peptide R16 (residues 1177-1191, ADGSSWEGVGVVPDV) for induction of EAU in Lewis rats were synthesized by GenScript.

2.2 Induction of EAU and experimental autoimmune encephalitis (EAE) in Lewis rats

EAU was induced in the Lewis rat either by active immunization with IRBP R16 peptide (aEAU) or transfer of R16-specific T cells (tEAU) using our previously reported procedures (19). For aEAU, the rats were injected subcutaneously with 100 □l of an emulsion containing 50 □g of R16 and 500 □g of *Mycobacterium tuberculosis* H37Ra in IFA, distributed over six spots at the tail base and on the flanks. For tEAU, recipient rats were injected intraperitoneally (ip) with in vitro re-stimulated 3 x 10^6 R16-specific T cells in 0.2 ml of PBS.

EAE was induced by immunization with 100□g/ml of myelin basic protein (MBP) from Guinee pig brain (Sigma) emulsified with 500 μg of H37Ra in IFA as described previously (20).

The rats were examined daily by a converted slit-lamp bio-microscope. Intensity of uveitis was scored blind on an arbitrary scale of 0 to 4 as previously described (19). Inflammation of the eye was confirmed by histopathology. Severity of EAU was

evaluated by masked colleagues and scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, as described previously (21).

2.3 Preparation of R16-specific T cells for tEAU

R16 specific T cells were isolated from R16-immunized Lewis rats using previously described methods (19). T cells were isolated from the draining lymph nodes and spleen at 14 days postimmunization (p.i.) by passage through a nylon wool column. The cells (1×10^7) were then stimulated for 2 days with 20 μg/ml of R16 in 2 ml of culture RPMI 1640 in a six-well plate (Corning) in the presence of 1×10^7 irradiated syngeneic spleen cells as APCs. Activated lymphoblasts were isolated by gradient centrifugation in Percoll Plus (GE Healthcare Life Sciences), counted and ip injected to naive Lewis rats (19)

2.4 Isolation of cells from inflamed eyes

After perfusion of the anesthetized rat with PBS on the indicated day after transfer of T cells, the eyes were collected and a cell suspension prepared by digestion for 10 min at 37°C with collagenase (1 mg/ml) and DNase (100 μg/ml) in RPMI 1640, followed by gradient centrifugation on 25% Percoll and subsequent Ficoll separation, then the cells were washed and resuspended in staining buffer (PBS containing 3% FCS and 0.1% sodium azide) for antibody staining (21).

2.5 Isolation of circulating exosomes

Circulating exosomes were isolated from peripheral blood of rats using a total exosome isolation (from plasma) kit (Cat# 4484450, Life technologies) following the manufacturer's instructions. All circulating exosomes in aEAU (EAU-Exo) were isolated on d14 post immunization (22). The identity of isolated exosomes was confirmed by electron microscopy (EM) and western blot using mAb against exosome marker CD63 (23). The concentration of exosomes was determined by their protein contents using a Bradford assay kit (Sigma). For Electronic microscopy examination, isolated pellets were fixed with 2% paraformaldehyde, loaded on Formvar-coated grids. The samples were

negatively stained with phosphotungstic acid for 1 minute and examined with Phillips CM10 electron microscopes at 72000x (23).

2.6 Vaccination of circulating exosomes

Three days prior to T cell injections, 500 □g of circulating exosomes in 100 □l of PBS were mixed with an equal volume of IFA and subcutaneously injected into 3-4 spots on the back of naïve rats.

2.7 T cell analysis by flow cytometry

For staining Foxp3+ Tregs, aliquots of 1×10^6 cells were incubated for 30 min at 4°C with anti-CD4 (Clone OX35) or isotype control antibodies, fixed overnight with 1 ml of fixation buffer, washed, and incubated for 30 min at 4°C with anti-Foxp3 (Clone 150D/E4) antibodies. For intracellular cytokine staining, cells were pretreated for 4 h with 50 ng/ml of phorbol myristic acetate (PMA), 1 □g/ml of ionomycin, and 1 □g/ml of brefeldin A (Sigma), then washed, fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience), and intracellularly stained with antibodies against IFN-□(Clone DB1) and IL-17 (Clone eBio17B7). Data collection and analysis were performed on a FACScaliber flow cytometer using CellQuest software (San Jose, CA).

2.8 T cell proliferation assay

Nylon wool-enriched T cells, prepared from aEAU or tEAU rats were seeded at 4×10^5 cells/well in 96-well plates and cultured at 37°C for 60 h in a total volume of 200 µl culture medium with or without 10 □g/ml of R16 in the presence of irradiated syngeneic spleen antigen presenting cells (APCs) (1×10^5). In some experiments, additional exosomes at different concentrations were given. (^{3}H)thymidine incorporation during the last 16 h, was assessed using a microplate scintillation counter (Packard). The proliferative response was expressed as the mean cpm ± standard deviation (SD) of triplicate determinations. We also used BrdU assay kits (Roche) for T cell proliferative response which was expressed as the mean OD ± SD for triplicate

samples or the proliferative stimulus index calculated as the ratio of the mean OD measured in the presence of the R16 to that in the absence of R16 (21).

2.9 Cytokines by ELISA

To measure cytokine production by responder T cells, supernatants were collected 48 h after T cell stimulation and assayed for IFN-γ and IL-10 using commercially available ELISA kits (R&D Systems).

2.10 Data analysis and statistics

Experiments were repeated at least three times. An unpaired Student's *t* test for two sets of data or one-way ANOVA Dunnet for three or more means at one time or repeated ANOVA for clinical score of uveitis was used for statistical analysis. A *P* value < 0.05 was considered as significant. Values determined to be significantly different from those for controls were marked with an asterisk in the figures.

3. RESULTS

3.1 Circulating exosomes from aEAU rats (EAU-Exo) inhibited R16-specific T cell proliferation

We collected exosomes on day 14 from the plasma of Lewis rats that had been immunized with R16, at the height of intraocular inflammation, to determine the role of circulating EAU-Exo (16). A T cell proliferative assay was performed with splenic T cells, EAU-Exo exosomes and 10 □g R16/ml. As shown in Fig 1, isolated T cells from aEAU rats proliferated in response to R16 in the absence of exosomes. However, EAU-Exo significantly inhibited R16 specific T cell proliferation in a dose-dependent manner.

3.2 Vaccination with EAU-Exo significantly inhibited the induction of tEAU

tEAU in rats is a recurrent uveitis model, resembling clinically recurrent uveitis in man (16,18). Since EAU-Exo inhibited R16-specific T cells in vitro, we hypothesized that vaccination with EAU-Exo might induce tolerance to R16-specific T cells. As seen in Fig

2A, vaccinated EAU-Exo Lewis rats developed tEAU by day 3 after adoptive transfer of IRBP T cells; however, they had fewer and less severe relapses compared to both control and Naïve-Exo vaccinated rats. Eye infiltrating cells isolated on day 21 of tEAU (Fig 2B & C) showed fewer CD4+ T cells (3.8±3.2 %) in vaccinated eyes compared to control eyes (10.7±2.6%). Both Th1 and Th17 cells were significantly reduced in the eye of vaccinated tEAU rats compared to control-treated tEAU rats. Furthermore, inhibition of tEAU was only observed when EAU-Exo were injected sc., but not ip or intravenously (iv) (data not shown).

3.3 EAU-Exo vaccination reduced IRBP-specific T cell proliferation and Th1 cytokine production, and increased IL-10 production

We measured R16-specific responses of T cells from the three groups of tEAU rats vaccinated in Fig 2A to explore the protective mechanism of EAU-Exo vaccination. Enriched T cells isolated from the spleens of these rats on day 21 after adoptive T cell transfer were stimulated with 10 □g/ml of R16 in vitro for 2 days. Vaccinated tEAU rats with EAU-Exo showed significantly decreased antigen specific T cell proliferation compared to control and Naïve-Exo vaccinated tEAU rats (Fig 3A). We also examined if vaccination affected IFN-γ autoreactive T cell secretion since antigen specific Th1 cells are important for tEAU induction. As shown in Fig 3B, levels of IFN-γ released into culture supernatants by T cells from EAU-Exo vaccinated-tEAU rats were markedly lower than those produced by T cells from control or Naive-Exo vaccinated tEAU rats, indicating an inhibitory effect of vaccination on pathogenic T cells.

Additionally, R16-specific T cells (Fig 3C) from EAU-Exo vaccinated tEAU rats produced significantly higher amounts of IL-10 in response to antigenic challenge compared to control and Naïve-Exo tEAU T cells. Examination of the phenotype of R16-specific T cells from the spleens of EAU-Exo vaccinated tEAU rats on day 21 showed that the percentage of CD4+Foxp3+ cells was not significantly different from control and Naïve-Exo tEAU rats (Fig 3D and E). Thus, vaccination with EAU-Exo suppressed the production of pro-inflammatory IFN-□and increased the production of anti-

inflammatory IL-10 from IRBP-specific T cells, leading to inhibition of intraocular inflammation.

3.4 EAU-Exo vaccination suppresses both T cell and APC function

Next, we examined whether the reduced proliferation of lymphocytes was a direct effect of EAU-Exo on T cells or an indirect effect via APCs. We performed crossover tests in which T cell proliferation was measured using all four combinations of responder T cells and APCs, isolated on day 21 from EAU-Exo vaccinated and control tEAU rats. As shown in Fig 3F, T cells from vaccinated tEAU rats did not respond to increasing doses of R16 in the presence of APCs from either vaccinated or control tEAU rats, whereas T cells from control tEAU rats reacted well in the presence of APCs from control tEAU rats but not vaccinated tEAU rats. These results suggest that dysfunction of both T cells and APCs contributed to the T cell hyporesponsiveness in EAU-Exo vaccinated rats.

3.5 EAU-Exo inhibition is antigen specific

To determine whether inhibition of EAU-Exo is antigen specific we induced both aEAU and aEAE in Lewis rats. Circulating exosomes and their splenic T cells on day 14 post-immunization were collected, and stimulated with their immunizing antigens, R16 or MBP, respectively. Their proliferation was determined using BrdU assay kits in the presence of increasing doses of EAU-Exo and EAE-Exo for 48 h. As shown in Fig 4A, T cells from aEAU rats proliferated to R16. This increase was inhibited by ≥10 □g/ml of EAU-Exo, but not EAE-Exo. Likewise, T cells from aEAE rats proliferated to MBP and were also inhibited by 10 □g/ml of EAE-Exo but not EAU-Exo (Fig 4B). Antigen specific inhibition was further confirmed in vivo since recurrent tEAU was only inhibited by EAU-Exo but not EAE-Exo (Fig 4C). The recurrence and severity of tEAU vaccinated with the same dose of EAE-Exo was comparable to tEAU controls. Histologic evaluation at the end of the experiment (day 61) showed infiltrating cells in the vitreous and severe retinal damage in the control and EAE-Exo-treated rats, but not in the EAU-Exo-treated group (Fig. 4D).

These results indicate that inhibition of EAU-Exo on R16-specific T cells was antigen specific.

4. DISCUSSION

Circulating exosomes can mediate immune responses and induce either immunogenic or tolerogenic responses in different environments. In autoimmune diseases such as MS in man (3) , and rodent models of autoimmune myocarditis (5), circulating exosomes promote autoreactive T cell responses. We assumed that circulating exosomes in autoimmune uveitis in rodents would also enhance autoreactive T cell immunity. However, our results show that circulating exosomes harvested from EAU recipients suppressed antigen-specific T cell immune responses in vitro. Furthermore, vaccination with EAU-Exo reduced the severity and relapses of adoptively transferred T cell-induced uveitis (i.e. tEAU) in an antigen-specific manner. Circulating exosomes inhibited APCs and antigen specific Th1 and Th17 cells, while increasing IL-10 production.

Circulating exosomes with immunosuppressive or tolerogenic effects have been observed in both patients and animal models (24). Circulating exosomes from patients with acute kidney injury (AKI) down-regulated miR-500a-3P, which suppressed cell injury and inflammation in human tubular epithelial cells (25). In cancer patients, serum-derived exosomes were reported to modulate T cell effector activity and promote tumor progression (26, 27). Plasma- or serum-derived exosomes isolated from antigen-fed animals were found to suppress Th1-dominated delayed-type hypersensitivity (DTH), as well as Th2-type allergic responses (28-30).Circulating exosomes isolated from mice immunized with keyhole limpet hemocyanin (KLH) antigen showed a potent suppressive effect on the KLH-induced DTH response after local administration, in part through a Fas/FasL-dependent process (22). The effect was antigen specific since plasma-derived exosomes isolated from mice immunized with an irrelevant antigen did not induce suppression. Our results in EAU showed similar antigen-specific inhibition.

EAU-Exo only inhibited IRBP-specific T cells but not MBP-specific T cells; likewise, EAE-Exo inhibited MBP-specific T cells but not IRBP-specific T cells. Our results also confirmed other findings observed with KLH-induced DTH – namely, optimal immunosuppressive activity was obtained with exosomes isolated 14 days after IRBP-immunization.

Circulating exosomes isolated from autoimmune diseases contain numerous autoantigens that may regulate immune responsiveness (31). Exosomes isolated from the synovial fluid of RA patients contain citrullinated proteins, which are known to be autoantigens (32). Additionally, exosomes containing autoantigens initiate and perpetuate autoantibody production in systemic autoimmune diseases such as SLE, Sjögren's syndrome and juvenile idiopathic arthritis (JIA). They can also activate autoreactive T cells in nonobese diabetic mice (31-34). Vaccination of tumor-derived exosomes, carrying tumor-associated antigens, can enhance the immune response to cancer (35). In contrast, exosome-based vaccines containing allergens prevented allergic reactions in animal models (36). To date, using autoantigen carrying circulating exosomes as a vaccine to induce immune tolerance to treat autoimmune disease has not been studied. However, numerous studies have reported that pre-treatment with soluble antigen alone or with liposomes can prevent induction of disease (37, 38). Our observation that circulating exosomes in rodent autoimmune uveitis can suppress recurrent intraocular inflammation following adoptive transfer of T cells suggests that individualized autoimmune suppression of intraocular inflammation may be possible. It also suggests the possibility of utilizing autologous plasma-derived exosomes therapeutically for intraocular inflammation in patients not responsive to corticosteroids or requiring more potent immunomodulatory therapy.

The mechanism of subcutaneous autoantigen containing exosomes in suppression of the immune response is not clear. Subcutaneous injection of soluble autoantigens, with or without liposomes, is thought to rapidly enter the lymphatic system with uptake of antigen by resident dendritic cells (DCs) in draining lymph nodes,

resulting in a tolerogenic signal to T cells. Tolerance may be induced by several means including T cell deletion, T cell anergy or induction of Tregs (38). EAU is mediated by Th1 and Th17 cell secretion of IFNγ and IL-17A, respectively (39, 40). Here, we show that IRBP-specific T cells from the spleen of EAU-Exo vaccinated rats express low IFN-□□and high IL-10 production. Additionally, IFN-□and IL-17 producing $CD4^+$ T cells were dramatically reduced in the eyes of vaccinated rats. Since the percentage of Treg cells is not significantly increased in vaccinated rodents, we assume that IL-10 was derived from Th1 and/or Th2 cells (41). IL-10 production has been shown to be important for EAU remission (42, 43).

In the present study, subcutaneous vaccination with EAU-Exo 3 days prior to IRBP-specific T cell adoptive transfer did not prevent the first episode of intraocular inflammation. This suggests that direct or indirect T cell inhibition takes up to 14 days for effective inhibition of effector/memory T cells and/or newly activated T cells by self-antigens released from damaged ocular tissues. Such a delay in immune responsiveness is common following vaccination with many immunogenic proteins/peptides.

Other non-circulating exosomes such as those derived from human mesenchymal stem cells (16, 17) or from ex-vivo-generated IL-35-producing regulatory B-cells (18) have been demonstrated to ameliorate EAU in rats and mice, respectively. However, using circulating exosomes from patients with recurrent uveitis, to treat themselves, will avoid potential infectious pathogens associated with cell culture, as well as variation in treatment efficacy because of instability in manipulated cells.

In summary, our study reported an inhibitory effect of circulating exosomes on T cells from rodents with EAU and confirmed that the tolerogenic properties of self-vaccination with these exosomes on recurrent episodes of intraocular autoimmune inflammation is profound. However, the precise mechanism of protection, duration of

protection, and molecular composition of EAU-Exo need to be determined before clinical application of this novel approach is implemented.

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FIGURE LEGENDS

Figure 1: Circulating exosomes from aEAU rats inhibited EAU T cell proliferation in a dose dependent manner. T cells (4 x 10^5/well) prepared from the spleens of R16-immunized Lewis rats (14 days p.i.) were stimulated with R16 (10 μg/ml) and APCs in the presence of increasing doses of circulating exosomes from aEAU (EAU-Exo) and naïve rats (Naïve-Exo) (n=3), and T cell proliferation was assessed by ^{3}(H)thymidine uptake. The data is presented as the mean of triplets ±SD. ***$p < 0.001$ compared with T cells stimulated with APC and immunizing peptide in the presence of Naive-Exo in one-way ANOVA. The experiment was one of three repeats.

Figure 2: Vaccination with EAU-Exo inhibited recurrent EAU in Lewis rats. A: Lewis rats were subcutaneously injected with 200 □l mixture of IFA and PBS (control), 500 □g Naive-Exo or EAU-Exo (n=6 rats/each group). Three days later EAU was induced by adoptive transfer of R16 specific T cells in all rats. Clinical scores were evaluated daily after tEAU induction, presented as the mean ± SD and analyzed by two-way ANOVA. ***p<0.0001. This was one representative of four individual experiments with 6 rats in each group of each experiment. B: Representative flow plots of eye infiltrating CD4+ T cells and CD4+ gated IFN-□+, IL-17+, and double positive cells analyzed on d21 post tEAU vaccination with EAU-Exo and PBS control; infiltrating cells were stained with antibodies against CD4, IFN-□ or IL-17. C. Percentage of CD4+ T cells in an eye, as well as the percentage of IFN-□$^+$ IL-17$^+$ and IFN-□$^+$IL-17$^+$ in total CD4+ cells. Data (mean ± SD) were pooled from two eyes per rat, 6 rats in each group; representing 2 replicates. *: p<0.05, **< p<0.01 and ***p<0.001 compared to the Ctrl group using an unpaired student t test.

Figure 3: Vaccination with EAU-Exo inhibited antigen-specific T cell proliferation and IFN-□ production, but increased IL-10 production. Rats were treated as in Fig 2A. Responder T cells prepared on day 21 from the three groups of tEAU were incubated without or with 10 □g/ml R16. After 48 h their proliferation was measured using (^{3}H)

thymidine uptake (A), and IFN-□(B) and IL-10 (C) in the supernatants were measured by ELISA. D: When the above rats in Fig 2A were sacrificed at day 21 after tEAU induction, splenocytes were stained with FITC conjugated CD4 and PE-conjugated Foxp3 Abs followed by flow cytometry analysis. E: Percentages of $CD4^+FoxP3^+$ cells in the spleen of each group. Data (mean ± SD) pooled from 6 rats in each group and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test, representing 2 replicates. F: Rats were treated as in Fig 2A, then responder T cells and splenic APCs were prepared on day 21 from tEAU vaccinated with EAU-Exo and PBS control. Proliferation of all four combinations of T cells and APCs in the stimulation with increasing doses of R16 were measured. Proliferation index represents T cells with antigen stimulation divided by T cell without antigen stimulation. All values are expressed as mean ±SD. **P<0.01 and ***p<0.001 compared to the Ctrl group. Data in A-C and F represent one experiment of three repeats.

Figure 4: Antigen specific inhibition of EAU-Exo on responding T cells and recurrent EAU. A and B: T cells prepared from the spleen of day 14 R16 (A) or MBP (B) immunized Lewis rats were stimulated with 10□g/ml of R16 (A) or MBP (B) and syngeneic irradiated spleen cells in the presence of graded doses of circulating exosomes isolated from d14 aEAU (EAU-Exo) or aEAE (EAE-Exo) Lewis rats. After 48 h, T cell proliferation was determined by BrdU assay. Data pooled from 3 rats, representing 2 replicates. C: Lewis rats were pre-vaccinated with 200 □l mixture of IFA and PBS (no Exo), 500 □g EAE-Exo or EAU-Exo. After 3 days, all rats were adoptive transferred with R16-specific T cells, clinical score was evaluated daily after tEAU induction (n=7 or 8 rats). Presented as mean ± SD and analyzed by two-way ANOVA. *p<0.05, and**p<0.01, representing 2 replicates. D: Mean ± SD of pathological scores of C at the end of experiments (D61 post-immunization) analyzed by one way-ANOVA followed by Tukey's multiple comparisons test. ***p<0.001.

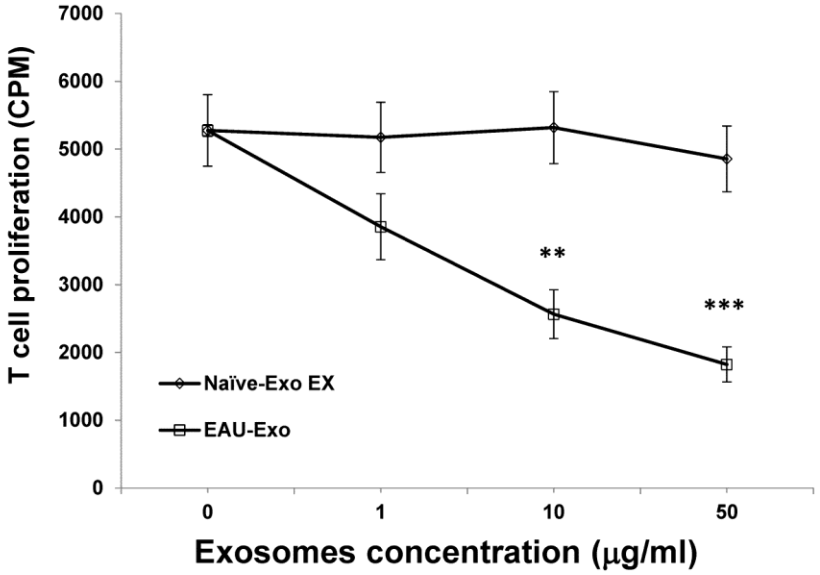

Fig 1

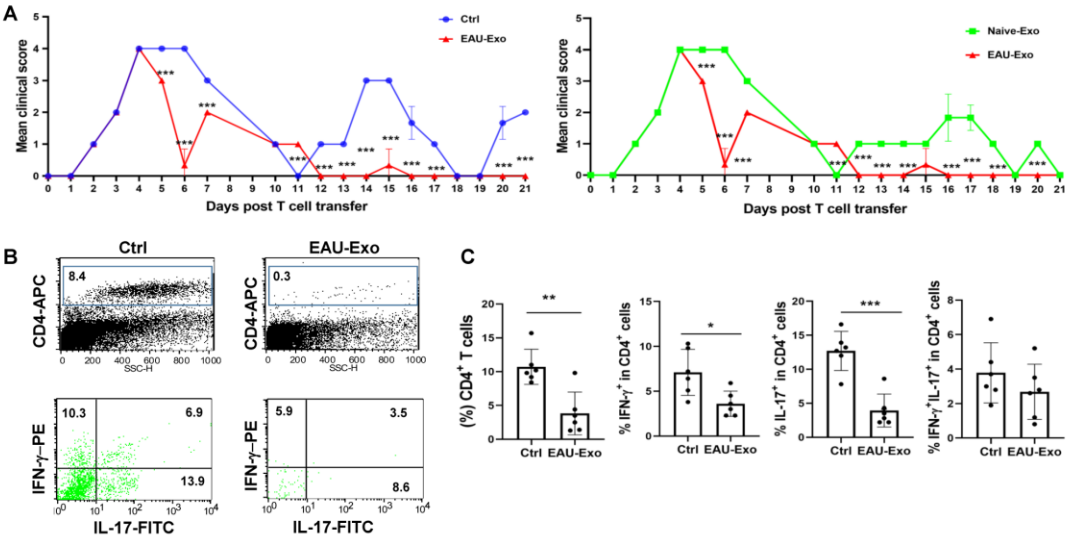

Fig 2

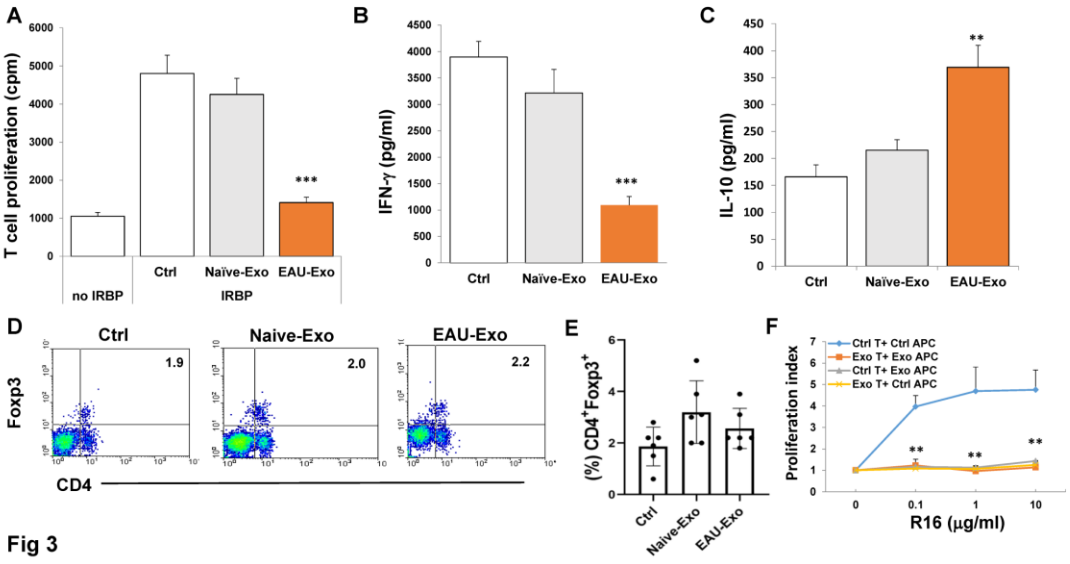

Fig 3

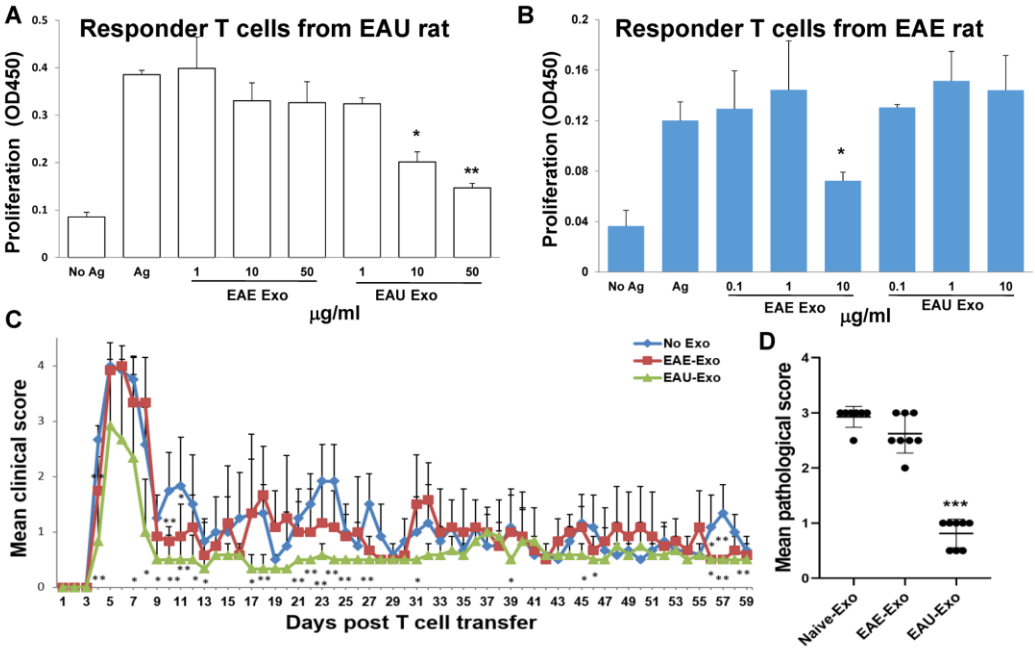

Fig 4